

## MOLECULAR ANALYSIS OF *SORBUS* SP. FROM THE PIENINY MTS. AND ITS RELATION TO OTHER *SORBUS* SPECIES

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The systematic position of *Sorbus* population occurring in the Pieniny Mts. is controversial. To verify its taxonomic status we studied the ITS sequence of closely related species of the *S. aria* group: *Sorbus* sp. from the Pieniny Mts., *S. aria* from the Tatra Mts., *S. graeca* from the Balkans, and other well-distinguished native Polish *Sorbus* species (*S. aria*, *S. aucuparia*, *S. intermedia* and *S. torminalis*). As a reference we examined *Sorbus* populations closest to the Pieniny Mts. where *S. graeca* was reported to occur, in Slovakia. The results indicate that the *Sorbus* plants found in the Pieniny Mts. differ genetically from those in the Tatra Mts. but are identical to those collected from the Vihorlat Mts. in Slovakia and are closely related to *S. graeca* from the Balkans.

**Key words:** *Sorbus*, Rosaceae, ITS, phylogeny, distribution, classification.

### INTRODUCTION

*Sorbus* is a genus comprising 250–300 species of trees and shrubs of the family Rosaceae, subfamily Maloideae (Phipps and Smith, 1990; Aldasoro et al., 2004; McAllister, 2005), occurring in the Northern Hemisphere. Recent classifications (Robertson et al., 1991; McAllister, 2005; Campbell et al., 2007; Potter et al., 2007) treat *Sorbus* in a narrower sense, including only pinnate-leaved species of subgenus *Sorbus* and raising several other subgenera to generic rank. In its broad sense the genus is divided into the following subgenera represented by diploid sexual type species: subgenus *Sorbus* (genus *Sorbus* s.str.) with compound leaves usually hairless or thinly hairy below, fruit carpels not fused (type species *Sorbus aucuparia*); subgenus *Aria* (genus *Aria*) with simple leaves usually strongly white-hairy below, fruit carpels not fused (type species *Sorbus aria*); subgenus *Micromeles* (genus *Aria*), an indistinct group of a few East Asian species with simple leaves (often included in subgenus *Aria*); subgenus *Cormus* (genus *Cormus*) with compound leaves similar to subgenus *Sorbus* but with distinctly fused carpels in the fruit (one species,

*Sorbus domestica*); subgenus *Torminaria* (genus *Torminalis*) with fruit carpels not fused (one species – *Sorbus torminalis*); and subgenus *Chamaemespilus* (genus *Chamaemespilus*) represented by the single species *Sorbus chamaemespilus* with simple, glabrous leaves. Besides these few sexual species, the genus *Sorbus* comprises a variety of taxa of hybrid, polyploid and apomictic origin. Apomixis played a crucial role in the evolution of the genus *Sorbus*, facilitating stabilization of hybrid forms (Jankun, 1993). It developed independently in three subgenera (*Sorbus*, *Aria*, *Chamaemespilus*) almost throughout its distribution range in Asia, Europe and North America (Jankun, 1993). *Sorbus* microspecies are found frequently in Europe and often are of very limited distribution; usually one of the parental species is *S. aria*. Such species are especially abundant in the British Isles, Germany, Scandinavia, the Czech Republic, Slovakia and Hungary. In Central Europe there are many other species, often poorly defined, variable, probably of hybrid origin, and of more or less widespread distribution, such as *S. pannonica*, *S. danubialis*, *S. austriaca*, *S. hazslinszkyana*, *S. hungarica* and *S. thaiszii* (Majovsky, 1992).

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One of the most intriguing *Sorbus* species in Poland is *Sorbus* growing in the Pieniny Mts., whose systematic position is unclear. The populations have been treated as *S. carpatica* and *S. aria* var. *incisa* (Pawłowska and Pawłowski, 1970), later as *S. graeca* (Gabriellán, 1976) or even as representatives of these three species and *S. austriaca* subsp. *hazslinszkyana* (Staszkievicz, 1997).

Traditional morphological taxonomy cannot clarify the systematic position of closely related *Sorbus* species. A molecular approach is required. Several molecular systematics techniques have been successfully applied to study selected *Sorbus* species: for example, RFLP (Nelson-Jones et al., 2002), chloroplast coding and noncoding DNA sequences (Campbell et al., 2007; Chester et al., 2007), nuclear ITS and starch synthase sequences (Campbell et al., 2007).

For these phylogenetic analyses we used DNA sequences of nuclear noncoding internal transcribed spacers (ITS) of rRNA determined for *Sorbus* from the Pieniny Mts., *S. aria* from the Tatra Mts., two *Sorbus* populations from stands in Slovakia where *S. graeca* was reported, *S. graeca* from the Balkan region, and three other well-defined native *Sorbus* species from Poland (*S. aucuparia*, *S. intermedia*, *S. torminalis*) for comparison.

Eukaryotic genes coding ribosomal RNA, rDNA occur in genomes as repeated units organized in tandems at nucleolar organizing regions (NORs). Every repeated unit comprises transcribed regions: 18S, 5.8S, 26S rRNA, ETS1 and ETS2 (external transcribed spacers), as well as nontranscribed spacers (NTS). Within the transcribed regions are ITS sequences flanking 5.8S rRNA on both sides (ITS1 and ITS2). The sequence of ITS rRNA regions undergoes rapid evolution and therefore is very useful in molecular phylogenetics (Feliner and Rosello, 2007). Due to their short length (600–750 bp) and high number of copies (up to 30,000 per cell) within a genome, ITS sequences are easily amplified. ITS sequences have been used in a number of phylogenetic and biogeographical studies (Baldwin et al., 1995; Gernandt et al., 2001; Alvarez et al., 2003; Ajani et al., 2008).

## MATERIALS AND METHODS

### PLANT MATERIAL

Leaves of the following species were obtained from natural stands: *S. aucuparia* – Małe Pieniny Mts. (2 plants); *S. 'Pieniny'* – Pieniny Mts. (Sokolica) (2 plants); *S. aria* – Tatra Mts. (Strążyska valley, Koński Żleb) (2 plants from 2 populations); *S. torminalis* – Białowodzka Góra Reserve (2 plants); *Sorbus* 'Biela Skala' – Biela Skala near

Krompachy (Slovakia) (3 plants); *Sorbus* 'Vihorlat' – Krivoštianka in Vihorlat Mts. (Slovakia) (1 plant); *S. intermedia* – Warsaw University Botanical Garden (2 plants), occurring naturally in the Baltic region; and *S. graeca* (= *S. umbellata* var. *cretica*) (Gabriellán, 1976) – Warsaw University Botanical Garden (2 plants), originating from the Balkan Peninsula. According to Dr. Vlastimil Mikoláš (Košice) (pers. comm.), the samples collected from Biela Skala are close to *S. javorkae* and *S. danubialis*, while those from Vihorlat can be ascribed to *S. danubialis* s.l.

### DNA EXTRACTION, CLONING AND SEQUENCE ANALYSIS

#### DNA isolation

Leaves (80 mg) were frozen in liquid nitrogen and homogenized in a mortar to obtain powder. DNA was isolated using a Genomic Mini AX Plant Spin kit (Aanda Biotechnology). Isolation yielded ~4.5 µg genomic DNA devoid of RNA and protein impurities.

#### Amplification of ITS regions

The primers employed for amplification of the ITS1-5.8S-ITS2 rDNA region were ITS 4 (5' TCCTC-CGCTTATTGATATGC3') and ITS 5 (5' GGAAGTAA-AAGTCGTAACAAGG3'). Amplification was performed in 100 µl reaction mixture containing 78 µl dH<sub>2</sub>O, 10 µl RUN 10× buffer, 2 µl 10 µM dNTP, 2 µl RUN polymerase (Taq polymerase from Aanda Biotechnology, activity 1 U/µl), 10 µM FOR and REV primers (2 µl each) and DNA template (20–100 ng). PCR conditions: initial denaturation, 5 min 95°C; 35 cycles of 1 min 95°C, 1 min 50°C, 1 min 72°C; terminal template elongation, 5 min 72°C. The obtained products were analyzed in agarose gel and the ~750 bp band corresponding to the ITS region was cut out. DNA was isolated from the gel using a Gel-out kit (Aanda Biotechnology).

#### Cloning of ITS products into pGEM®-T Easy vector

The purified PCR products (ITS1-5.8S-ITS2 rDNA fragments) were cloned into pGEM®-T Easy vector according to the manufacturer's instructions. Cloning the PCR product into the vector prevents errors in ITS nucleotide sequence determination. Ligation was performed at 16°C. The ligation mixture was used for transformation of competent bacteria *E. coli* DH5α. Then the transformants were identified by blue/white screening. The presence of the insert in white bacterial colonies was confirmed by PCR. Primers M13 FOR and M13 REV complementary to the sequences within pGEM®-T Easy vector were applied. PCR conditions: initial denatura-

TABLE 1. Polymorphism of the ITS sequences of the investigated *Sorbus* species. Polymorphic nucleotide sites are indicated using the IUPAC code: R = G or A, Y = T or C.

Species	Position in the alignment																							
	11	33	111	114	119	125	131	152	155	205	209	343	345	466	509	523	533	538	561	563	569	586		
<i>S. aria</i>	A	G	R	C	G	GC	C	Y	C	G	T	G	T	T	G	T	T	C	C	C	C	G		
<i>S. 'Pieniny'</i>	A	G	R	C	G	GC	C	Y	C	G	T	G	T	T	G	T	T	C	C	C	C	G		
<i>S. graeca</i>	A	G	R	C	G	GC	C	Y	C	G	T	G	T	T	G	T	T	C	C	C	C	G		
<i>S. aucuparia</i>	A	A	G	A	A	GC	T	G	A	A	T	A	T	C	A	T	C	T	T	T	T	A		
<i>S. torminalis</i>	G	T	G	C	G	TT	C	G	C	G	A	G	C	C	G	C	T	T	C	T	C	A		
<i>S. intermedia</i>	A	A	G	A	A	TT	T	G	A	A	T	A	T	C	A	C	C	T	T	T	T	A		

tion, 5 min 95°C; 29 cycles of 1 min 95°C, 1 min 53°C, 1.15 min 72°C; terminal template elongation, 5 min 72°C.

#### Sequencing

Clones containing the appropriate inserts were cultured overnight, followed by plasmid DNA isolation using a Plazmid Mini kit. Sequencing was performed by GENOMED (Poland). Commercially available primers T7 For and SP6 Rev were used. Up to six clones of each transformant were sequenced, containing ITS1-5.8S-ITS2 rDNA fragments originating from each of the *Sorbus* plants examined. Intraindividual polymorphism did not vary between the plants examined. The obtained sequences were aligned using BLAST (<http://blast.ncbi.nlm.nih.gov>) or SRS@EBI (N ClustalW) (<http://srs.ebi.ac.uk>).

#### MOLECULAR PHYLOGENETIC ANALYSIS

The analysis was based on the ITS sequences of *Sorbus* species determined in the samples collected from natural stations and the botanical garden (see above). As outgroup we used the ITS sequence from *Geum urbanum* U90802, and the ITS sequence from *S. caloneura* FJ810008 (subgenus *Micromeles*) as reference (GenBank, [www.ncbi.nlm.nih.gov](http://www.ncbi.nlm.nih.gov)).

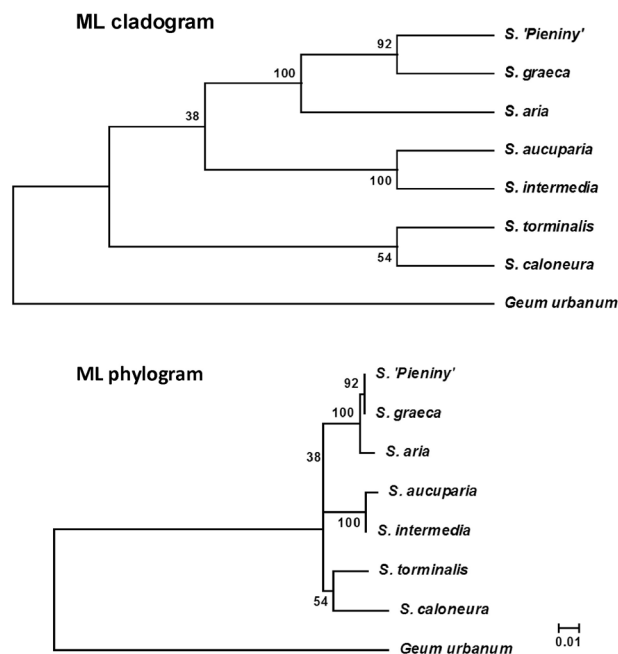
All sequences were aligned with ClustalW implemented in MEGA5 (Tamura et al., 2011). The default parameters were used for alignment of the sequences: gap opening penalty 15.0, gap extension penalty 6.66, delay divergent sequences (%) 30. The IUB scoring matrix was used for comparing ITS sequences and the default transition weight was 0.5.

The maximum likelihood (ML) method was based on the Tamura 3-parameter model (Tamura, 1992). The tree with the highest log likelihood (-1485.7122) is shown. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (100 replicates) is shown next to the branches (Felsenstein, 1985). Initial tree(s) for the heuristic search were obtained automatically

by applying the neighbor-joining and BioNJ algorithms to a matrix of pairwise distances estimated by the maximum composite likelihood (MCL) approach, and then selecting the topology with superior log likelihood value. A discrete Gamma distribution was used to model evolutionary rate differences between sites (2 categories [+G, parameter = 0.0500]). The tree is drawn to scale, with branch lengths measured in the number of nucleotide substitutions per site. All positions containing gaps and missing data were eliminated. There were 580 positions in the final dataset. Evolutionary analyses were conducted in MEGA5 (Tamura et al., 2011).

#### RESULTS AND DISCUSSION

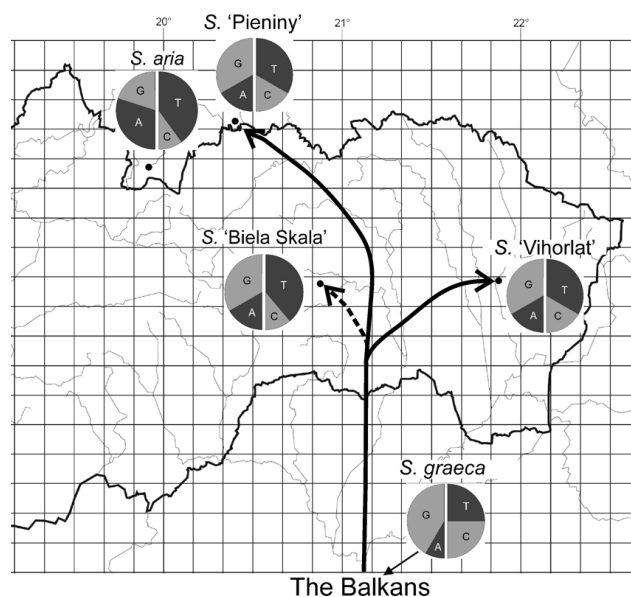
For phylogenetic analysis we used ITS sequences for four well defined *Sorbus* species native to the Polish flora (*S. aria* from the Tatra Mts., *S. torminalis*, *S. intermedia*, *S. aucuparia*), *Sorbus* sp. from the Pieniny Mts. and *S. graeca* from the Balkans. The length of ITS sequences determined for the species, including the 5.8S rRNA sequence (ITS1-5.8S rRNA-ITS2), was 699–702 bp. Alignment revealed 71 polymorphic sites, 20 of which were informative (Tab. 1). The phylogenetic trees based on the obtained sequences constructed using the maximum likelihood method are shown in Figure 1. The species of *Sorbus aria* agg. (*S. aria*, *Sorbus* 'Pieniny', *S. graeca*), belonging to subgenus *Aria*, are grouped together in both the cladogram and the phyllogram. *Sorbus* from the Pieniny Mts. is evidently more closely related to *S. graeca* from the Balkans than to *S. aria* from the Tatra Mts. *Sorbus torminalis*, morphologically a clearly distinct species (subgenus *Torminaria*), is located on a separate branch together with *S. caloneura*, a representative of sect. *Micromeles*, used here as a reference. *S. aucuparia* and *S. intermedia* form a different clade. The close position of *S. intermedia* and *S. aucuparia* (Fig. 1) is an interesting result, since *S. intermedia* is regarded as a hybrid of



**Fig. 1.** Molecular phylogenetic analysis of the investigated *Sorbus* species using the maximum likelihood method based on ITS sequences. Scale represents expected number of substitutions per nucleotide position. Next to branches is the percentage of replicate trees in which the associated taxa clustered, by the bootstrap test (100 replicates). *Geum urbanum* ITS sequence was used as out-group. For further details see Materials and Methods.

*S. aria*, *S. aucuparia* and *S. torminalis* in several works (Jankun, 1993; Nelson-Jones et al., 2002; Heupler and Muer, 2007). The two species occupied a similar position on the phylogenetic tree of the subtribe Pyrinae (Li et al., 2012). The sequences of *S. intermedia* and *S. torminalis* show unique common nucleotides (positions 125, 126 and 523 in Tab. 1), indicating their genetic relation.

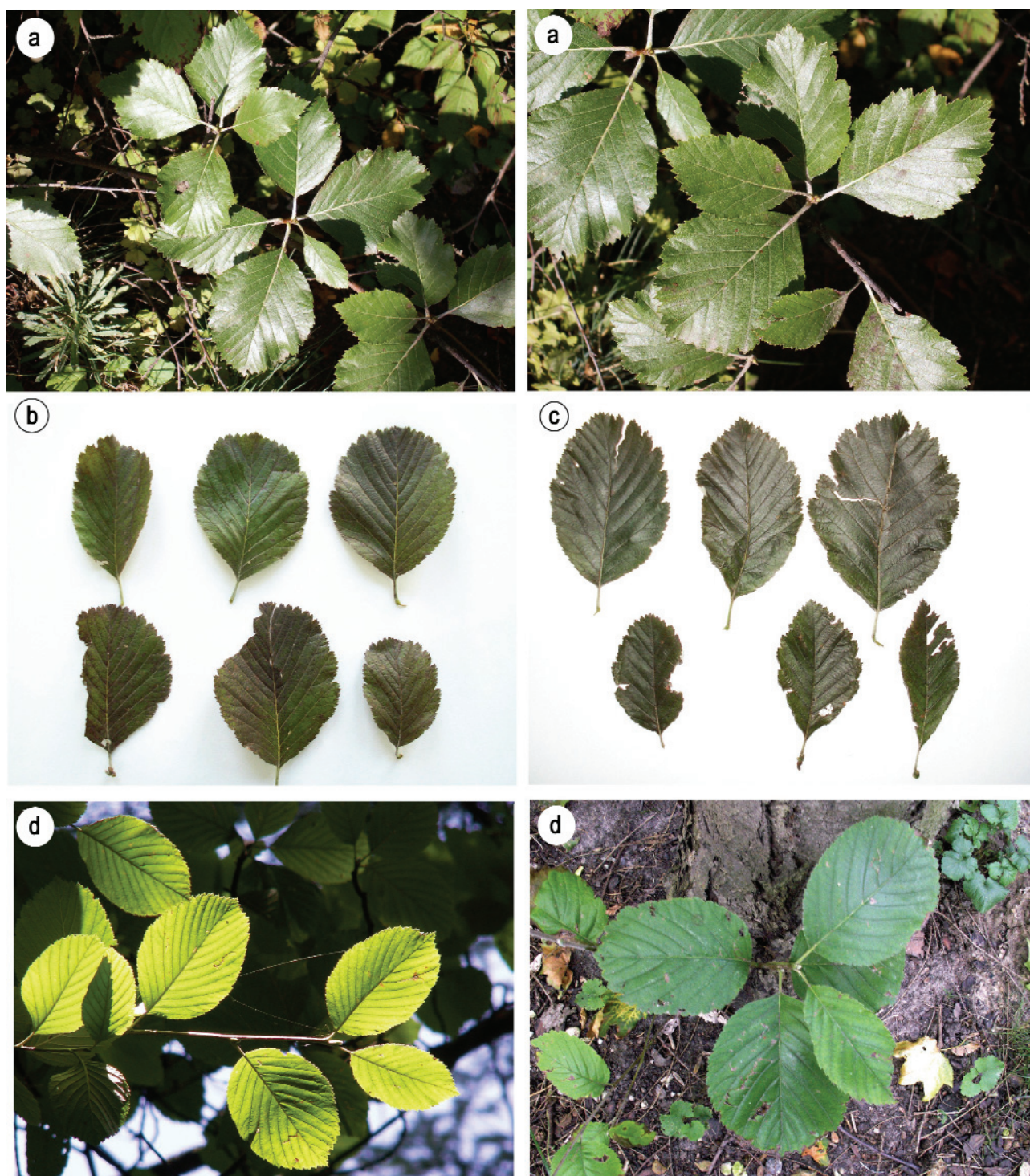
In the sequence analysis we included *Sorbus* specimens from Slovakia, collected from sites where *S. graeca* was reported (Vihorlat Mts., Biela Skala) (Majovsky, 1992; see Materials and Methods for species determination). The ITS sequence comparison for these plants revealed polymorphism at 23 sites, only two of which were useful for phylogenetic analysis (111 and 152 in Tab. 1), where the analyzed species of the *S. aria* group showed differences in the nucleotides. These sites showed intraindividual polymorphism (Fig. 2). The diagrams indicate that the percentage of the nucleotides at the first analyzed position (111) was the same for *Sorbus* from the Pieniny Mts. and the localities in Slovakia, and were closer to *S. graeca* from the Balkans than to *S. aria* from the Tatra Mts. Analysis of the second polymorphic site (152) indicates that the popula-



**Fig. 2.** Distribution and phylogeography of the investigated species of the *S. aria* group: *S. graeca* from the Balkans, *S. aria* from the Tatra Mts., *Sorbus* sp. from the Pieniny Mts. and two populations of *Sorbus* from Slovakia. Figure indicates the polymorphism of nucleotides (frequency of indicated nucleotides) at positions 111 (left part of circle) and 152 (right part of circle) shown in Table 1. Black dots indicate localities of the investigated species, described in more detail in Materials and Methods.

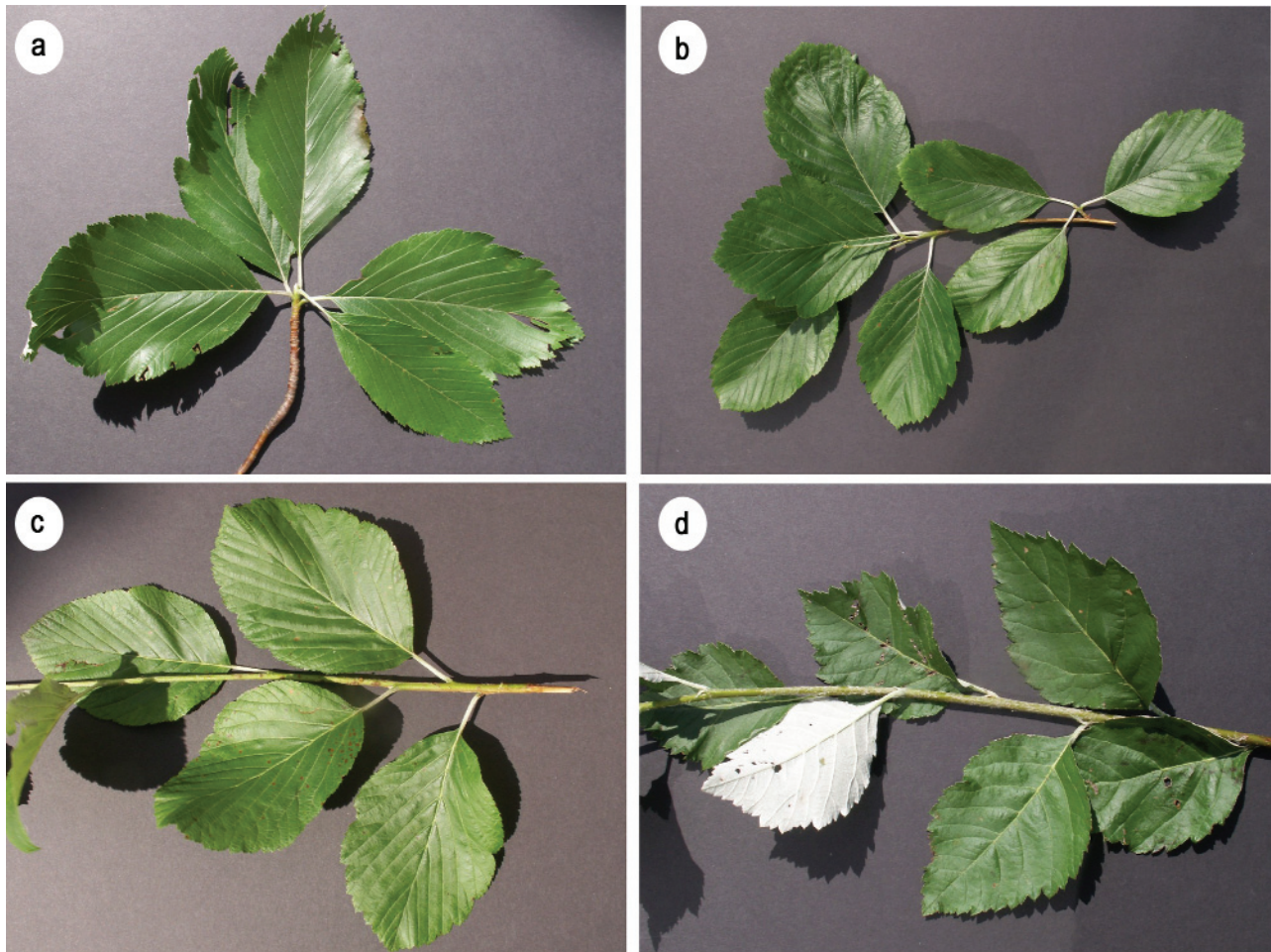
tions from the Pieniny and Vihorlat Mts. are genetically uniform and closer to *S. graeca* than to the plants from the Tatra Mts. and Biela Skala, which showed the same percentage of nucleotides at that position (Fig. 2). The results suggest that the *Sorbus* population from the Pieniny Mts. differ genetically from the one in the Tatra Mts. and is the same as the one in the Vihorlat Mts. (Fig. 2). The analyzed population from Biela Skala is intermediate between *S. aria* and *Sorbus* 'Pieniny' (Fig. 2). *Sorbus* from the Pieniny Mts. and from Slovakia differ genetically from the plants occurring in the Balkans, as indicated by the higher G content at the first analyzed position and the higher C content at the second position in *S. graeca*. Differences between the *Sorbus* species from the Pieniny Mts. and *S. graeca* are also evident in the morphology of the leaves (Fig. 3). *S. graeca* leaves are round and have finely serrate margins, while those from the Pieniny Mts. are of similar shape but have coarsely serrate margins in the upper part. The leaves of *S. aria* from the Tatra Mts. are round to elliptic, with rough margins mainly in the upper part. Leaf shape and number of nerves at each side are differences between *S. aria* and *S. graeca* mentioned in the literature





**Fig. 3.** Leaves of the investigated *S. aria* group species: (a) *Sorbus* sp. from Pieniny Mts., (b) *S. aria* from Strążyska valley and (c) Koński Żleb in Tatra Mts., (d) *S. graeca* from the Balkans. For detailed localities of the stands see Materials and Methods.





**Fig. 4.** Leaves of the investigated species from Slovakia. *Sorbus* 'Biela Skala' (a, b, c), *Sorbus* 'Vihorlat' (d).

(Rutkowski, 2011). For *S. graeca* it is reported that the leaves are oval to elliptic, with 7–9 (10) nerves on each side, while in *S. aria* the leaves are elliptic and have more nerves (10–14). In our study, however, the leaves of all the species had 9–10 nerves on each side and were of similar shape (Fig. 3). Interestingly, Pawłowska and Pawłowski (1970) classified plants with leaf shape similar to those shown in Figure 3a, collected in the Tatras and Pieniny Mts., as *S. carpatica*, and others from the Tatra Mts. with more ellipsoidal leaves as *S. aria* var. *aria* or *S. aria* var. *incisa*. The taxonomic status of *Sorbus* populations in the Tatra Mts. requires further study.

## CONCLUSIONS

1. The *Sorbus* populations from the Pieniny Mts. are genetically distant from those found in the Tatra Mts. but very close to the *Sorbus* species from Slovakia, especially from the Vihorlat

Mts., but neither population is identical to *S. graeca* from the Balkans.

2. The populations from the Pieniny Mts., where *S. graeca* reaches the northern limit of its entire range in Europe, are allied to it or to a taxon closely related to it. The possible migration route of *S. graeca* from the Balkans to the investigated area is shown schematically in Figure 2.
3. ITS sequence analysis is a useful method for investigating closely related species of the genus *Sorbus* but the differences between their ITS sequences are small, so other markers such as nontranscribed ITS sequences should be used in further research.

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